Cell Stem Cell
Minireview

Epigenetic Alterations in Human Pluripotent Stem Cells: A Tale of Two Cultures

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Human somatic cells can be reprogrammed into induced pluripotent stem cells (hiPSCs) with wide lineage differentiation potential in culture. However, reprogramming and long-term culture can also induce abnormalities in these pluripotent cells. This minireview discusses recent studies that have identified changes in imprinted gene expression and erosion of X chromosome inactivation in female hiPSCs and how understanding the sources and consequences of epigenetic variability in hiPSCs will impact disease modeling and clinical application in the future.

The ability to reprogram human somatic cells to an early embryonic state, known as induced pluripotent stem cell (iPSC) reprogramming, is a relatively new and powerful technology that allows researchers to easily generate large numbers of cells with the potential to differentiate into a wide range of cell types (Takahashi et al., 2007; Yu et al., 2007). Recently, the application of patient-derived hiPSCs for pharmacologic testing of chemical compounds in models of human disease, including long QT syndrome (Itzhaki et al., 2011), RETT syndrome (Marchetto et al., 2010), and Machado-Joseph disease (Koch et al., 2011), has been reported. Reprogramming results in a pluripotent phenotype that is believed to be largely identical to that of embryonic stem cells isolated from human embryos (hESCs). This view is supported by studies investigating their differentiation potential in culture and in teratoma formation assays. Although some genetic and epigenetic differences between hESCs and hiPSCs had been previously described (reviewed in Panopoulos et al., 2011), the potential repercussions for subsequent applications of iPSCs remained unclear. Recent studies have further characterized gene expression and epigenetic patterns of hiPSC lines in unprecedented detail. In this minireview, I will discuss these recent investigations, which reveal frequent aberrations in genomic imprinting and X chromosome inactivation (XCI), and their potential impact on the future application of hiPSC lines (Anguerra et al., 2012; Mekhoubad et al., 2012; Nazor et al., 2012). This brief review is not intended to be a comprehensive assessment of gene imprinting or XCI, and we refer readers to other, more complete reviews on these topics here (Augui et al., 2011; Ferguson-Smith, 2011).

Epigenetic Differences in hiPSCs

To examine the extent of epigenetic differences in hiPSCs and the potential impact for their use, Nazor et al. recently investigated gene expression and DNA methylation patterns in a set of over 200 hESC and hiPSC lines (Nazor et al., 2012). From these data, two groups of genes with reciprocal epigenetic patterns in the undifferentiated versus differentiated state were identified, including (1) a set of genes that is consistently methylated in hiPSCs and hESCs and unmethylated in all examined tissues, and (2) a group of genes that is methylated in hiPSCs and hESCs and is unmethylated only in specific tissues. A number of genes known to be expressed in a cell-type-specific manner belong to this latter group. Importantly, the lineage-specific demethylation could be recapitulated by directed differentiation, showing that developmental regulation of these genes is preserved in hiPSCs. This observation supports the validity of hiPSCs as models for development and disease and further suggests that demethylation of genes has a prominent function in differentiation. The large scope of this study also provided a unique opportunity for comprehensive analyses of variation between the hiPSC and hESC lines. A large set of genes was found to be variably methylated among different hESCs and hiPSCs, and this variable DNA methylation appeared to be predominantly associated with imprinted genes and genes on the X chromosome in female hiPSCs. Comparison of early and late passage hiPSCs indicated that while some changes in DNA methylation were likely results of the reprogramming process, others could be attributed to continuous growth in culture. Notably, specific culture methods seem to be associated with imprinting aberrations, such as hypermethylation of *DIRAS3*, which could be linked to serum containing culture media (Nazor et al., 2012). These findings are consistent with earlier observations of gene- and clone-specific changes of imprinting in hiPSCs (Pick et al., 2009). In addition, variable DNA methylation was observed in female hiPSC and hESC lines on X-linked genes that are unmethylated in male cells, thus reflecting the presence of an inactive X chromosome (Xi) in female cells. Variation was largely due to a loss of DNA methylation of X-linked genes that is associated with length in culture and correlates with elevated expression of genes on the Xi and a loss of *XIST* expression. These observations indicate that maintenance of XCI is compromised in female hiPSCs and hESCs at later passages.

Instability of the Xi was also observed in a study investigating female hiPSCs from patients with Lesch-Nyhan syndrome who carry a mutation of *HPRT* on one of their X chromosomes (Mekhoubad et al., 2012). In low-passage hiPSCs, inactivation of one of the two X chromosomes was observed and was derived from the Xi of the donor fibroblasts. Random XCI in the donor cell population gives rise to two types of hiPSC lines that express either the mutated or the intact *HPRT* gene, and analyzing pairs of mutant and control hiPSCs can be useful for

studying phenotypic differences caused by the mutation in the same genetic background. However, after prolonged passage, hiPSCs lose *XIST* expression, which was followed by reactivation of genes on the Xi. Reactivation of substantial portions if not the entire Xi could be demonstrated in some hiPSC lines. As a consequence *HPRT* expression was restored to heterozygous levels in hiPSC lines that were initially *HPRT* deficient, making these cells unsuitable for modeling Lesch-Nyhan syndrome. Importantly, neither differentiation nor further reprogramming appeared to restore *XIST* expression or XCI. This finding suggests that erosion of dosage compensation is a permanent aberration in late-passage female hiPSCs.

In this issue of *Cell Stem Cell*, an independent study confirms *XIST* expression and a cytologically distinct XCI in early-passage hiPSCs (Anguerra et al., 2012). *XIST* expression is also observed to be frequently lost upon prolonged culture, consistent with an erosion of Xi silencing. Genome-wide expression profiling further demonstrates that the expression of X-linked, but not autosomal, genes is frequently elevated in late-passage female hiPSCs, which leads to expression profiles with signatures that have been implicated in cancer. Specifically, loss of *XIST* expression is correlated with upregulation of X-linked oncogenes along with an accelerated growth rate in vitro (Anguerra et al., 2012). Disruption of XCI is further associated with an impaired differentiation potential. Taken together, these studies show that erosion of DNA methylation and gene repression on the Xi of female hiPSCs occurs during long-term culture and affects disease modeling and differentiation potential. In addition to female-specific aberrations in XCI, errors in imprinted gene expression should now be considered in male and female hiPSCs.

Tomoda et al. use a different culture system for reprogramming human cells (Tomoda et al., 2012). This system is based on a proprietary medium and includes SNL feeder cells (McMahon and Bradley, 1990) that express the cytokine LIF, which is important for the culture of mouse ESCs. Female hiPSCs derived under these conditions possess an Xi at early passages, consistent with results of other studies (Anguerra et al., 2012; Mekhoubad et al., 2012). However, the Xi is efficiently reactivated upon further culture, and two active X chromosomes are observed. In this study, Xi reactivation requires LIF and is not observed in conditions where LIF or SNL feeder cells are omitted. Reactivation of the Xi is accompanied by a loss of *XIST* and higher expression of X-linked genes. Importantly, XCI is initiated and dosage compensation is restored upon differentiation. This observation is important because it shows that the epigenetic state of these cells differs from iPSCs cultured in the other studies.

Different Developmental States of Human Pluripotent Cells

All four studies agree on the point that early-passage hiPSCs possess an Xi, thus reflecting maintenance of XCI from the somatic donor cells. However, the dissimilarities reveal that several different processes can lead to changes in epigenetic characteristics, as observed by loss of Xi silencing. Whereas Tomoda et al. observe that XCI is restored upon differentiation of their hiPSCs, the other studies observe irreversible erosion of dosage compensation (Anguerra et al., 2012; Mekhoubad et al., 2012; Nazor et al., 2012). These studies follow earlier work that has led to conflicting observations. Studies of female hiPSCs from RETT patients carrying a heterozygous X-linked mutation in the *MECP2* gene have observed either Xi reactivation (Marchetto et al., 2010) or maintenance of XCI from the somatic donor cells after reprogramming (Pomp et al., 2011). To reconcile these differences it is helpful to consider findings from reprogramming in the mouse. Mouse somatic cells can be reprogrammed into two different types of pluripotent stem cells, corresponding to the preimplantation and postimplantation embryo state (Han et al., 2011). Standard culture conditions for hESCs are not conducive for mouse ESC culture but have been used to derive a second pluripotent cell type from the mouse postimplantation epiblast known as epiblast stem cells (EpiSCs; Brons et al., 2007; Tesar et al., 2007). Mouse EpiSCs have cell signaling and transcriptional characteristics similar to hESCs. Notably, XCI is observed in female mouse EpiSCs, whereas ESCs possess two active X chromosomes (Guo et al., 2009). This has led to the proposal that mouse ESCs represent an earlier developmental state commonly referred to as ground state or naive pluripotency. This distinction is functionally relevant as mouse ESCs, but not EpiSCs, have the potential to contribute extensively to development of mice upon blastocyst injection or tetraploid embryo complementation. In contrast mouse EpiSCs can form a wide range of cell types in culture but inefficiently contribute to development.

This raises the question of whether human pluripotent cells with properties similar to mouse ESCs can be obtained. Several reports have applied modified mouse ESC culture conditions for establishing hESCs and hiPSCs with properties of mouse ESCs (Buecker et al., 2010; Hanna et al., 2010; Lengner et al., 2010; Wang et al., 2011b), albeit, ground state hiPSCs in some of these studies were metastable (Buecker et al., 2010), required expression of additional factors (Wang et al., 2011b), or were limited in passage (Hanna et al., 2010). These findings strongly suggest that similar to the situation in the mouse, two distinct types of human pluripotent stem cells might be cultured.

In hindsight, discrepancies on the XCI status of hiPSCs can partly be reconciled by considering differences in culture conditions. Loss of Xi gene silencing can result from either erosion of dosage compensation or reactivation of the Xi due to capturing a different pluripotent cell type (Figure 1). Similar to hiPSCs, female hESCs vary in their XCI status and a useful classification has been suggested (Silva et al., 2008). Class I hESCs possess two active X chromosomes and lack *XIST* expression. Upon differentiation, *XIST* expression is observed and XCI is initiated, thus resembling properties of mouse ESCs indicative of ground state pluripotency. Class II hESCs have initiated XCI and express *XIST*. This class seems to correspond to early-passage hiPSC cultures (Anguerra et al., 2012; Mekhoubad et al., 2012; Tchieu et al., 2010; Tomoda et al., 2012). Class III hESCs are characterized by the presence of an Xi but have lost *XIST* expression. Importantly, XCI is not initiated when class III hESCs are induced to differentiate. These class III hESCs show similarities to late-passage hiPSCs with irreversible erosion of dosage compensation (Anguerra et al., 2012; Mekhoubad et al., 2012; Nazor et al., 2012). Depending on the choice of culture conditions, the ratio of cells of the three classes can vary. In addition, cells of late-passage class II or class III cultures do not appear to revert easily

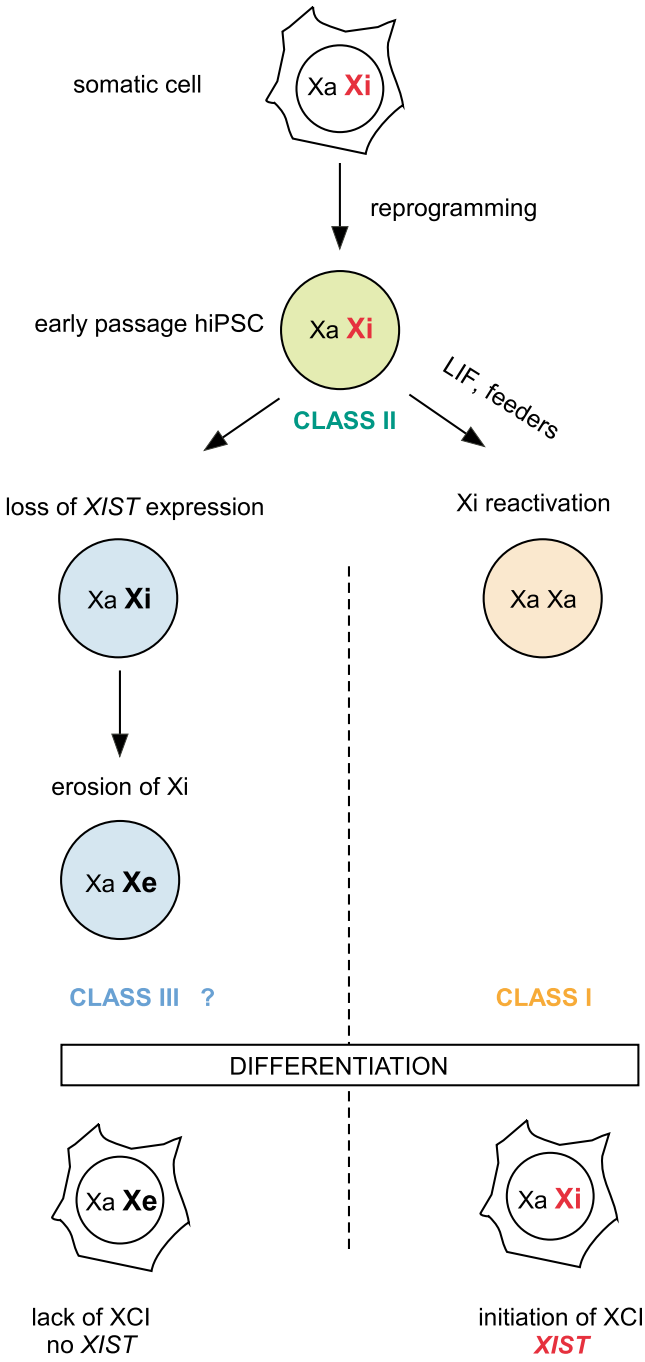

Figure 1. Effect of Culture on XCI in Female hiPSCs

XCI is maintained from the somatic donor cell in early-passage hiPSCs. These class II cells express *XIST*, and a cytologically distinct Xi can be detected. Characteristics of the hiPSCs change upon further culture, depending on the conditions. In the presence of LIF and feeder cells, Xi reactivation associated with loss of *XIST* is observed. The resulting class I cells resemble aspects of ground state pluripotency and will undergo random XCI upon differentiation. Under standard culture condition loss of *XIST* and erosion of gene silencing on the Xi (Xe) is observed. Erosion of dosage compensation is irreversible and XCI is not initiated when these cells enter differentiation, similar to class III hESCs. *XIST* expression is indicated in red text.

to class I cells irrespective of the conditions used (Tomoda et al., 2012). Heterogeneity of epigenetic states in ESC cultures has also been observed in studies analyzing gene expression in single cells (Hayashi et al., 2008; Hough et al., 2009). Taken together, these data suggest that the culture history is an overarching factor for hiPSC characteristics.

The mouse system may provide further opportunities for exploring certain aspects of the human reprogramming process. Indeed, chemical inhibitors have been developed that facilitate the conversion of mouse EpiSCs to ground state pluripotent ESCs (Zhou et al., 2010). A recent study has further shown that epigenetic disruptions during reprogramming of mouse iPSCs can be prevented by ascorbic acid (Stadtfeld et al., 2012), which is a cofactor for histone demethylases (Wang et al., 2011a). In addition, the use of chemical inhibitors of histone deacetylases has been shown to influence epigenetic properties of mouse ESCs and hESCs (Hayashi et al., 2008; Ware et al., 2009). Importantly, these findings may be useful for obtaining hiPSCs that are less prone to phenotypic drift and epigenetic aberrations.

Culture Conditions for hESCs and hiPSCs

It is possible that phenotypic drift in culture is an inherent property of hESCs and hiPSCs. Alternatively, drift could be the result of culture-induced mutations (Cheng et al., 2012) or reflect limitations in culture conditions that could induce stress (Newman and Cooper, 2010; Nishino et al., 2011; Pera and Tam, 2010). The controversy over the endogenous state of XCI in hiPSCs and hESC has provided information on culture conditions that are either associated with XCI or not (Table 1). The study by Tomoda et al. shows that LIF is critical for ground state pluripotency in human cells (Tomoda et al., 2012). This finding has important implications for the interpretation of earlier studies that used conventional hiPSC culture conditions, which do not include LIF, and thus might observe cells evolving along a different trajectory (Anguerra et al., 2012; Nazor et al., 2012; Tchieu et al., 2010). LIF is crucial for maintenance of mouse and rat ESCs (Nichols and Smith, 2009). It is unclear how to interpret studies that investigate potential class I hiPSC (XaXa) maintenance in the absence of LIF in the culture medium. The expectation would be that exit from ground state pluripotency would be favored even if initially a sizeable population of class I cells were present. LIF is used to derive pre-XCI hESCs in low-oxygen conditions (Lengner et al., 2010). However, LIF is not included in the study by Anguerra et al. (in their experiments showing that the effect of low oxygen for Xi reactivation in hiPSCs cannot be recapitulated; Anguerra et al., 2012). Although this consideration cannot explain the discrepancy completely, it might be helpful for reconciling the different observations. Similarly, discrepant results on XCI in hiPSCs from RETT syndrome patients could be the result of different culture conditions (Marchetto et al., 2010; Pomp et al., 2011). Marchetto et al. use proprietary media that might favor ground state pluripotent hiPSCs, whereas Pomp et al. use standard hESC conditions. Importantly, when previously reported culture conditions for inducing ground state hiPSCs were applied (Hanna et al., 2010), Pomp et al. also observed Xi reactivation and subsequent random XCI in hiPSCs.

Adjusting the culture environment and reprogramming techniques has great potential for further improvements in hiPSC

Table 1. Selected Studies Examining either Ground State or EpiSC-like Human Pluripotent Cells

Pluripotent State	Culture Conditions	Properties Examined	References
mESC-like ground state	primate ESC medium (ReproCELL); SNL feeders (STO line expressing mLIF); 4 ng/ml bFGF, 33 ng/ml IGF2	XaXa - > XaXi (dif); gene expression	(Tomoda et al., 2012)
	N2B27-based medium; MEF feeders; hLIF, 10 μM forskolin; 3 μM CHIR99021, 1 μM PD0325901	XaXa - > XaXi (dif); Oct4 enhancer, gene expression, GF response	(Hanna et al., 2010)
	DMEM/F12; MEF feeders; hLIF, 15 ng/ml hFGF2; FBS/KOSR/plasmonate; physiological 4% oxygen	XaXa - > XaXi (dif); also XaXe observed in this study	(Lengner et al., 2010)
	mTeSR1 medium (StemCell Technologies) Matrigel	XaXa - > XaXi (dif); MECP2 mutation	(Marchetto et al., 2010)
	DMEM/F12; STO feeder cells; hLIF, 20% KOSR; 5 μM CHIR99021, 1 μM PD0325901	XaXa - > XaXi (dif); GF response	(Wang et al., 2011b)
	DMEM/F12; MEF feeders; 5 ng/ml bFGF, 20% KOSR (hLIF)	metastable hiPSCs; transgene-dependent ground state	(Buecker et al., 2010)
mEpiSC-like human ESCs	DMEM/F12; MEF feeders; 20 ng/ml FGF, 10% KOSR	XaXi and XaXe; gene expression	(Anguerra et al., 2012)
	standard conditions (FGF); MEF feeders; + rescue with mTeSR medium	XaXi and XaXe	(Mekhoubad et al., 2012)
	DMEM/F12; MEF feeders; 20 ng/ml bFGF, 20% KOSR	XaXi; gene expression	(Tchieu et al., 2010)
	KO-DMEM (Invitrogen); MEF feeders; 4 ng/ml bFGF, 20% KOSR	XaXi; MECP2 mutation; also validates Hanna et al. protocol for Xi reactivation	(Pomp et al., 2011)

Details of culture conditions, experimental evidence, and literature references are given. KOSR, serum replacement (Invitrogen).

quality. A number of informative studies reveal common media components that correlate with ground state pluripotency or Xi reactivation in hESCs and hiPSCs (Table 1; Figure 2). One surprising observation is the use of LIF and FGF for hiPSC culture, which have antagonistic effects on mouse ESCs (Nichols and Smith, 2009). FGF is used for the culture of mouse EpiSCs but has been linked to induction of differentiation of mouse ESCs. FGF seems to be a growth or survival signal for hiPSCs/hESCs and might at the same time induce EpiSC fate, thus favoring exit from ground state pluripotency. High amounts of FGF could result in EpiSC-like properties in hiPSCs, whereas lower levels in combination with Igf2 or insulin might act as a prosurvival and/or proliferation signal when differentiation is inhibited by LIF (Tomoda et al., 2012). Furthermore, it is interesting to note that LIF is not a complete substitute for SNL feeders in the study by Tomoda et al. Feeders could potentially contribute additional signals to maintain ground state pluripotency. Ground state pluripotency of mouse and rat ESCs can be maintained in the absence of feeders when MEK and GSK3 kinase activity is inhibited by chemical compounds (Nichols and Smith, 2009). These inhibitors have also been applied for obtaining ground state pluripotent hiPSCs (Hanna et al., 2010; Wang et al., 2011b). Notably, differences in FGF signaling in mouse and human embryos have also been observed, suggesting that species-specific differences might exist (Kuijk et al., 2012; Roode et al., 2012). It will be interesting to determine if combinations of FGF and LIF with GSK3 and MEK inhibition are useful for defining conditions for feeder-independent hiPSC culture.

Reprogramming Factor Expression

The stoichiometry and timing of expression of transcription factors during reprogramming is also an important factor for the characteristics of iPSCs. Suboptimal ratios in expression levels of reprogramming factors have been linked to a loss of imprinting at the *Dlk1-Dio3* locus in mouse iPSCs and have further been shown to compromise their ability to form all-iPSC-derived mice (Carey et al., 2011). Recent findings also suggest that additional reprogramming factors can lead to faster and more efficient reprogramming (Wang et al., 2011b). Conversely, removal of reprogramming vectors after reprogramming has been reported to enhance the differentiation potential of iPSCs (Sommer et al., 2010). Modest expression of viral *Klf4* is observed in some hiPSC lines generated by Mekhoubad et al. and might contribute to phenotypic drift (Mekhoubad et al., 2012). This is consistent with the observed persistent differences in gene expression signatures between hiPSCs and hESCs (Chin et al., 2009). For avoiding mutations caused by the integration of viral vectors, strategies for vector excision or the use of non-integrating vectors can be considered. Tomoda et al. use an episomal vector system for expressing reprogramming factors that might contribute to improving the quality of hiPSCs for disease modeling (Tomoda et al., 2012). However, vector insertions cannot explain the variability that is observed among hESC lines, suggesting that other factors are equally important.

X Inactivation in the Embryo and ESCs

One critical question is how similar ESC cultures are to the related cell types in the embryo. XCI has been studied extensively

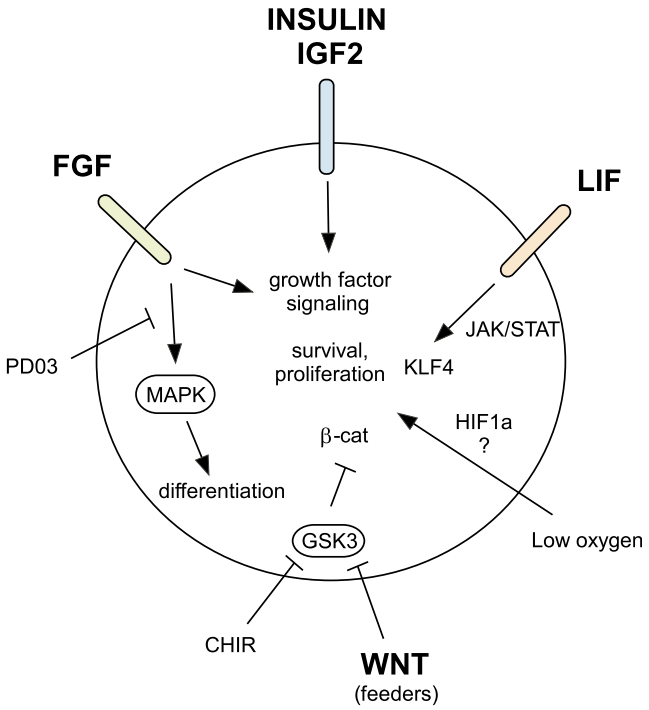

Figure 2. Culture Conditions Associated with Ground State Human Pluripotency

A number of studies have established hESCs and hiPSCs with properties similar to mouse ESCs. FGF, insulin, and IGF2 are thought to enhance proliferation, survival, and self-renewal of hESCs. The cytokine LIF activates the JAK/STAT signaling pathway and induces transcription factors, including Klf4, in mouse ESCs. A combination of chemical inhibitors of GSK3 (CHIR) and MEK (PD03) kinase activity has been used to capture ground state pluripotency in hiPSCs. MAPK signaling has been associated with differentiation. Inhibition of GSK3 kinase activity by chemical inhibitors or WNT signaling leads to stabilization of β-catenin (β-cat) that has been implicated in ESC self-renewal through inactivation of TCF3 repressor function. In addition low oxygen levels support ground state pluripotency of hESCs.

in the mouse embryo where inactivation of the paternal X chromosome is initiated at the four-cell stage. The Xi is then reactivated in the ICM of the blastocyst at E4.5 followed by initiation of random XCI in the E5.5 embryo. Thus, mouse ESCs correspond to the ICM and early epiblast, whereas EpiSCs might represent features of postimplantation epiblast cells.

A recent study shows that XCI is not initiated in the human embryo at the blastocyst stage (Okamoto et al., 2011). The reported isolation of pre-XCI hESCs under low-oxygen conditions is consistent with this observation (Lengner et al., 2010). However, an earlier study had found that XCI is already initiated in cleavage-stage human embryos (van den Berg et al., 2009). These conflicting results are hard to reconcile and could point to subtle differences in embryo culture or genetic variation. Human preimplantation development takes longer and is more complex than that of mice (reviewed in Niakan et al., 2012), suggesting that the epigenetic states of cells at the blastocyst stage might not be as clearly defined as in the mouse (Kuijk et al., 2012; Roode et al., 2012). Potentially, this could also contribute to the variability of XCI in hESCs and hiPSCs.

Nazor et al. make the important observation that many genes are methylated in hESCs and hiPSCs but are found in an unmethylated state in tissues (Nazor et al., 2012). It is not clear if these methylation patterns reflect the presumed developmental state of hESCs. The current view is that DNA methylation is reduced in the pluripotent cells of the embryo and increases upon further development. This raises the question of whether culture-induced hypermethylation could contribute to the observed DNA methylation patterns in hiPSCs.

Conclusion and Future Outlook

Epigenetic aberrations have been noticed in earlier studies of hiPSCs, but the implications for the application of hiPSCs remained unclear (Panopoulos et al., 2011). The present studies now demonstrate that epigenetic aberrations occur frequently in hiPSCs and limit their use for disease modeling and potential clinical applications. In particular, the reduced differentiation potential and tumorgenicity induced by erosion of Xi silencing in female hiPSCs is a concern for future applications of these cells (Anguerra et al., 2012). A crucial question is what triggers the phenotypic and epigenetic changes in hiPSCs. Loss of *XIST* under standard hiPSC culture conditions is not easy to explain but could be the result of stress-induced changes. This view is supported by the observation that fibroblasts isolated from RETT patients acquire a skewed XCI ratio before reprogramming (Pomp et al., 2011), suggesting that culture stress is a concern. Similar stress might be present in hiPSC cultures but could be harder to detect. The identified instability and drift in XCI and genomic imprinting might provide a sensitive readout for further optimizing culture conditions.

It appears paramount to avoid epigenetic aberrations during reprogramming and subsequent culture. Changing the developmental state of hiPSC cultures might provide additional opportunities to reach this aim. The use of FGF in standard hiPSC culture conditions as a mitogen is likely to also induce an EpiSC-like phenotype in hiPSCs. These cultures could be prone to drift with progressive changes in DNA methylation and loss of XCI. The hiPSC derivation by Tomoda et al. appears to lead to more stable cultures. However, using SNL feeders is reminiscent of the early mouse ESC culture before chemically defined conditions became available. Increasing understanding of human and mouse pluripotent states will facilitate the development of chemically defined culture conditions that are a prerequisite for clinical use of hiPSCs.

At the moment it remains unclear if ground state pluripotent hiPSCs might be less exposed to epigenetic aberrations and drift. The competence of ground state mouse and rat ESCs to contribute to embryogenesis suggests that stable culture conditions that maintain a well-preserved developmental potential can be achieved (Nichols and Smith, 2009). However, comprehensive analysis of epigenetic and phenotypic properties of ground state hiPSC lines will need to be confirmed in the future using the genomic technologies as outlined in the study by Nazor et al. Notably, Tomoda et al. find that XCI is initiated when their hiPSCs are differentiated into endodermal cells, but *XIST* expression was not observed (Tomoda et al., 2012). This is unusual because *XIST* expression is a marker for XCI in other systems. This peculiarity might be linked to the particular choice of differentiation protocol. Future investigation of other differentiation protocols will be important especially because Hanna et al. have shown that a shift to culture media with serum and

FGF suffices to induce *XIST* in ground state hiPSCs (Hanna et al., 2010).

Maintaining XCI in female hiPSCs may be advantageous for modeling X-linked disease such as RETT syndrome where female patients are heterozygous for the disease-causing mutation. It is conceivable that ground state hiPSCs could be converted into neural stem cell cultures that have defined XCI patterns to facilitate disease modeling. Understanding the biology and characteristics of hiPSCs will certainly benefit future clinical applications in regenerative medicine as well as research into disease mechanism. Using powerful genomics technology will provide the needed resolution to facilitate the development of improved methods for reprogramming human cells.

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REFERENCES

Anguerra, M., Sadreyev, R., Zhang, Z., Szanto, A., Payer, B., Sheridan, S., Kwok, S., Haggarty, S.J., Sur, M., Alvarez, J., et al. (2012). Molecular signatures of human induced pluripotent stem cells (hiPSCs) highlight sex differences and cancer genes. Cell Stem Cell *11*, this issue, 75–90.

Augui, S., Nora, E.P., and Heard, E. (2011). Regulation of X-chromosome inactivation by the X-inactivation centre. Nat. Rev. *12*, 429–442.

Brons, I.G., Smithers, L.E., Trotter, M.W., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S.M., Howlett, S.K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R.A., and Vallier, L. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature *448*, 191–195.

Buecker, C., Chen, H.H., Polo, J.M., Daheron, L., Bu, L., Barakat, T.S., Okwieka, P., Porter, A., Gribnau, J., Hochedlinger, K., and Geijsen, N. (2010). A murine ESC-like state facilitates transgenesis and homologous recombination in human pluripotent stem cells. Cell Stem Cell *6*, 535–546.

Carey, B.W., Markoulaki, S., Hanna, J.H., Faddah, D.A., Buganim, Y., Kim, J., Ganz, K., Steine, E.J., Cassady, J.P., Creyghton, M.P., et al. (2011). Reprogramming factor stoichiometry influences the epigenetic state and biological properties of induced pluripotent stem cells. Cell Stem Cell *9*, 588–598.

Cheng, L., Hansen, N.F., Zhao, L., Du, Y., Zou, C., Donovan, F.X., Chou, B.K., Zhou, G., Li, S., Dowey, S.N., et al; NISC Comparative Sequencing Program. (2012). Low incidence of DNA sequence variation in human induced pluripotent stem cells generated by nonintegrating plasmid expression. Cell Stem Cell *10*, 337–344.

Chin, M.H., Mason, M.J., Xie, W., Volinia, S., Singer, M., Peterson, C., Ambartsumyan, G., Aimiuwu, O., Richter, L., Zhang, J., et al. (2009). Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. Cell Stem Cell *5*, 111–123.

Ferguson-Smith, A.C. (2011). Genomic imprinting: the emergence of an epigenetic paradigm. Nat. Rev. *12*, 565–575.

Guo, G., Yang, J., Nichols, J., Hall, J.S., Eyres, I., Mansfield, W., and Smith, A. (2009). Klf4 reverts developmentally programmed restriction of ground state pluripotency. Development *136*, 1063–1069.

Han, D.W., Greber, B., Wu, G., Tapia, N., Araúzo-Bravo, M.J., Ko, K., Bernemann, C., Stehling, M., and Schöler, H.R. (2011). Direct reprogramming of fibroblasts into epiblast stem cells. Nat. Cell Biol. *13*, 66–71.

Hanna, J., Cheng, A.W., Saha, K., Kim, J., Lengner, C.J., Soldner, F., Cassady, J.P., Muffat, J., Carey, B.W., and Jaenisch, R. (2010). Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. Proc. Natl. Acad. Sci. USA *107*, 9222–9227.

Hayashi, K., Lopes, S.M., Tang, F., and Surani, M.A. (2008). Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states. Cell Stem Cell *3*, 391–401.

Hough, S.R., Laslett, A.L., Grimmond, S.B., Kolle, G., and Pera, M.F. (2009). A continuum of cell states spans pluripotency and lineage commitment in human embryonic stem cells. PLoS ONE *4*, e7708.

Itzhaki, I., Maizels, L., Huber, I., Zwi-Dantsis, L., Caspi, O., Winterstern, A., Feldman, O., Gepstein, A., Arbel, G., Hammerman, H., et al. (2011). Modelling the long QT syndrome with induced pluripotent stem cells. Nature *471*, 225–229.

Koch, P., Breuer, P., Peitz, M., Jungverdorben, J., Kesavan, J., Poppe, D., Doerr, J., Ladewig, J., Mertens, J., Tüting, T., et al. (2011). Excitation-induced ataxin-3 aggregation in neurons from patients with Machado-Joseph disease. Nature *480*, 543–546.

Kuijk, E.W., van Tol, L.T., Van de Velde, H., Wubbolts, R., Welling, M., Geijsen, N., and Roelen, B.A. (2012). The roles of FGF and MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. Development *139*, 871–882.

Lengner, C.J., Gimelbrant, A.A., Erwin, J.A., Cheng, A.W., Guenther, M.G., Welstead, G.G., Alagappan, R., Frampton, G.M., Xu, P., Muffat, J., et al. (2010). Derivation of pre-X inactivation human embryonic stem cells under physiological oxygen concentrations. Cell *141*, 872–883.

Marchetto, M.C., Carromeu, C., Acab, A., Yu, D., Yeo, G.W., Mu, Y., Chen, G., Gage, F.H., and Muotri, A.R. (2010). A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. Cell *143*, 527–539.

McMahon, A.P., and Bradley, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell *62*, 1073–1085.

Mekhoubad, S., Bock, C., de Boer, A.S., Kiskinis, E., Meissner, A., and Eggan, K. (2012). Erosion of dosage compensation impacts human iPSC disease modeling. Cell Stem Cell *10*, 595–609.

Nazor, K.L., Altun, G., Lynch, C., Tran, H., Harness, J.V., Slavin, I., Garitaonandia, I., Müller, F.J., Wang, Y.C., Boscolo, F.S., et al. (2012). Recurrent variations in DNA methylation in human pluripotent stem cells and their differentiated derivatives. Cell Stem Cell *10*, 620–634.

Newman, A.M., and Cooper, J.B. (2010). Lab-specific gene expression signatures in pluripotent stem cells. Cell Stem Cell *7*, 258–262.

Niakan, K.K., Han, J., Pedersen, R.A., Simon, C., and Pera, R.A. (2012). Human pre-implantation embryo development. Development *139*, 829–841.

Nichols, J., and Smith, A. (2009). Naive and primed pluripotent states. Cell Stem Cell *4*, 487–492.

Nishino, K., Toyoda, M., Yamazaki-Inoue, M., Fukawatase, Y., Chikazawa, E., Sakaguchi, H., Akutsu, H., and Umezawa, A. (2011). DNA methylation dynamics in human induced pluripotent stem cells over time. PLoS Genet. *7*, e1002085.

Okamoto, I., Patrat, C., Thépot, D., Peynot, N., Fauque, P., Daniel, N., Diabangouaya, P., Wolf, J.P., Renard, J.P., Duranthon, V., and Heard, E. (2011). Eutherian mammals use diverse strategies to initiate X-chromosome inactivation during development. Nature *472*, 370–374.

Panopoulos, A.D., Ruiz, S., and Izpisua Belmonte, J.C. (2011). iPSCs: induced back to controversy. Cell Stem Cell *8*, 347–348.

Pera, M.F., and Tam, P.P. (2010). Extrinsic regulation of pluripotent stem cells. Nature *465*, 713–720.

Pick, M., Stelzer, Y., Bar-Nur, O., Mayshar, Y., Eden, A., and Benvenisty, N. (2009). Clone- and gene-specific aberrations of parental imprinting in human induced pluripotent stem cells. Stem Cells *27*, 2686–2690.

Pomp, O., Dreesen, O., Leong, D.F., Meller-Pomp, O., Tan, T.T., Zhou, F., and Colman, A. (2011). Unexpected X chromosome skewing during culture and reprogramming of human somatic cells can be alleviated by exogenous telomerase. Cell Stem Cell *9*, 156–165.

Roode, M., Blair, K., Snell, P., Elder, K., Marchant, S., Smith, A., and Nichols, J. (2012). Human hypoblast formation is not dependent on FGF signalling. Dev. Biol. *361*, 358–363.

Silva, S.S., Rowntree, R.K., Mekhoubad, S., and Lee, J.T. (2008). X-chromosome inactivation and epigenetic fluidity in human embryonic stem cells. Proc. Natl. Acad. Sci. USA *105*, 4820–4825.

Sommer, C.A., Sommer, A.G., Longmire, T.A., Christodoulou, C., Thomas, D.D., Gostissa, M., Alt, F.W., Murphy, G.J., Kotton, D.N., and Mostoslavsky, G. (2010). Excision of reprogramming transgenes improves the differentiation potential of iPS cells generated with a single excisable vector. Stem Cells *28*, 64–74.

Stadtfeld, M., Apostolou, E., Ferrari, F., Choi, J., Walsh, R.M., Chen, T., Ooi, S.S., Kim, S.Y., Bestor, T.H., Shioda, T., et al. (2012). Ascorbic acid prevents loss of Dlk1-Dio3 imprinting and facilitates generation of all-iPS cell mice from terminally differentiated B cells. Nat. Genet. *44*, 398–405, S391–392.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*, 861–872.

Tchieu, J., Kuoy, E., Chin, M.H., Trinh, H., Patterson, M., Sherman, S.P., Aimiuwu, O., Lindgren, A., Hakimian, S., Zack, J.A., et al. (2010). Female human iPSCs retain an inactive X chromosome. Cell Stem Cell *7*, 329–342.

Tesar, P.J., Chenoweth, J.G., Brook, F.A., Davies, T.J., Evans, E.P., Mack, D.L., Gardner, R.L., and McKay, R.D. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature *448*, 196–199.

Tomoda, K., Takahashi, K., Leung, K., Okada, A., Narita, M., Yamada, N.A., Eilertson, K.E., Tsang, P., Baba, S., White, M.P., et al. (2012). Derivation conditions determine X-reactivation status in female human induced pluripotent stem cells. Cell Stem Cell *11*, this issue, 91–99.

van den Berg, I.M., Laven, J.S., Stevens, M., Jonkers, I., Galjaard, R.J., Gribnau, J., and van Doorninck, J.H. (2009). X chromosome inactivation is initiated in human preimplantation embryos. Am. J. Hum. Genet. *84*, 771–779.

Wang, T., Chen, K., Zeng, X., Yang, J., Wu, Y., Shi, X., Qin, B., Zeng, L., Esteban, M.A., Pan, G., and Pei, D. (2011a). The histone demethylases Jhdm1a/1b enhance somatic cell reprogramming in a vitamin-C-dependent manner. Cell Stem Cell *9*, 575–587.

Wang, W., Yang, J., Liu, H., Lu, D., Chen, X., Zenonos, Z., Campos, L.S., Rad, R., Guo, G., Zhang, S., et al. (2011b). Rapid and efficient reprogramming of somatic cells to induced pluripotent stem cells by retinoic acid receptor gamma and liver receptor homolog 1. Proc. Natl. Acad. Sci. USA *108*, 18283–18288.

Ware, C.B., Wang, L., Mecham, B.H., Shen, L., Nelson, A.M., Bar, M., Lamba, D.A., Dauphin, D.S., Buckingham, B., Askari, B., et al. (2009). Histone deacetylase inhibition elicits an evolutionarily conserved self-renewal program in embryonic stem cells. Cell Stem Cell *4*, 359–369.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., et al. (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science *318*, 1917–1920.

Zhou, H., Li, W., Zhu, S., Joo, J.Y., Do, J.T., Xiong, W., Kim, J.B., Zhang, K., Scholer, H.R., and Ding, S. (2010). Conversion of mouse epiblast stem cells to an earlier pluripotency state by small molecules. J. Biol. Chem. *285*, 29676–29680.